OFFICE OF NAVAL RESEARCH

Contract No. ONR N00014-76-C-0053
(15)
Task No. NR 204-002

(9) ANNUAL REPORT NO. 5, 31 Dec 80 - 1 Jan 81

(6) Penetration Enzymes of Schistosome Cercariae,

by

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31 December 1980 (11) 1 Jan 81 (12) 21

PENETRATION ENZYMES OF SCHISTOSOME CERCARIAE

Annual Report
January 1, 1980 — December 31, 1980

N00014-76-C-0053

BACKGROUND

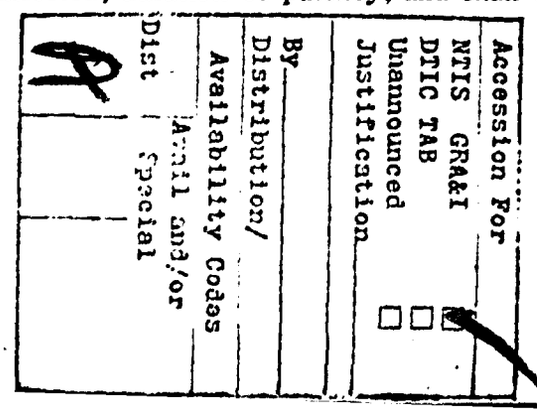
Free-swimming schistosome cercariae penetrate the tegument of the vertebrate host. Their infectivity, that is, their success in penetrating skin and subsequently maturing, is related to various conditions to which they are subjected during (1) their development in the snail, (2) their activity in the free-swimming environment, and (3) their adjustment to their postpenetration milieu. During this phase of the life cycle, several steps occur about which our knowledge is incomplete: cercariae emerge with variable infective potentials; penetrated skin is altered; the parasite undergoes extensive developmental changes; and immunological reactions are stimulated. It is probably during this time that the parasite is both susceptible to immune attack and involved in the stimulation of protective immunity. It follows that understanding the details and ramifications of the process by which cercariae infect their hosts and the means of modifying this process are fundamental to work aimed at protecting hosts against infection, which is the purpose of this contract. Studies have been conducted on 4 aspects of this program.

1. Effect of Different Snail Exposure Levels on Parasite Development in Snails.

It is necessary to know what snail exposure levels best maintain development of the parasite and provide the largest cercarial collections most efficiently.

METHODS

Previously, 8-10, 6-8, and 1 miracidium per snail have been compared in this regard. To complete this series, for 8 consecutive weeks 150 snails of the Nmri line measuring the usual diameter (5 to 7 mm) were exposed individually each week under the same conditions either to 8-10 or to 5 miracidia each. From onset of patency to death of the snails, cercariae were collected twice a week. Records were kept on a total of 2400 snails: percentage of exposed snails which became infected; duration of patency; and snail deaths during both prepatent and patent periods.



SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER: 5	2. GOVT ACCESSION NO.: AD-A095 046	3. RECIPIENT'S CATALOG NUMBER:
4. TITLE (and Subtitle): PENETRATION ENZYMES OF SCHISTOSOME CERCARIAE		5. TYPE OF REPORT & PERIOD COVERED: Annual: 31 Dec 1980-1 Jan 1981 6. PERFORMING ORG. REPORT NUMBER:
7. AUTHOR(s): M. Stirewalt and F. A. Lewis		8. CONTRACT OR GRANT NUMBER(s): ONR N00014-76-C-0053
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Amer. Foundation for Biological Research, 12111 Parklawn Drive, Rockville, Maryland 20852		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: NR 204-002
11. CONTROLLING OFFICE NAME AND ADDRESS: Procurement Contract Officer, Office of Naval Research (443) Dept. of the Navy, Washington, D. C. 20360		12. REPORT DATE: 1 Jan 1981 13. NUMBER OF PAGES: 17
14. MONITORING AGENCY NAME & ADDRESS (If different from Controlling Office)		15. SECURITY CLASS. (of this report): Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Schistosoma mansoni; Biomphalaria glabrata; trematode; cercariae; schistosomules; optimal maintenance; ultrastructure; immune response; schistosome strains; infectivity; worms.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Cercarial production and rates of snail infection and death were similar from exposures of 8-10, 6-8 or 5 miracidia per snail. 5-miracidium exposures were most efficient. Snails limit number of primary sporocysts which develop regardless of number of miracidia. Different combinations of intraspecific geographical strains of S. mansoni and Biomphalaria glabrata varied in capability for cercarial production. Vaccination potentials were similar. Artificially transformed schistosomules lagged 48 to 96 hr behind in vivo in morphological and physiological changes.

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RESULTS

Average daily cercarial production is graphed in Figure 1. The overall average for 5-miracidium snails was 2582; for 8-10-miracidium snails, 2323. Reducing the number of miracidia to which each snail was exposed from 8-10 to 5 did not reduce cercarial production.

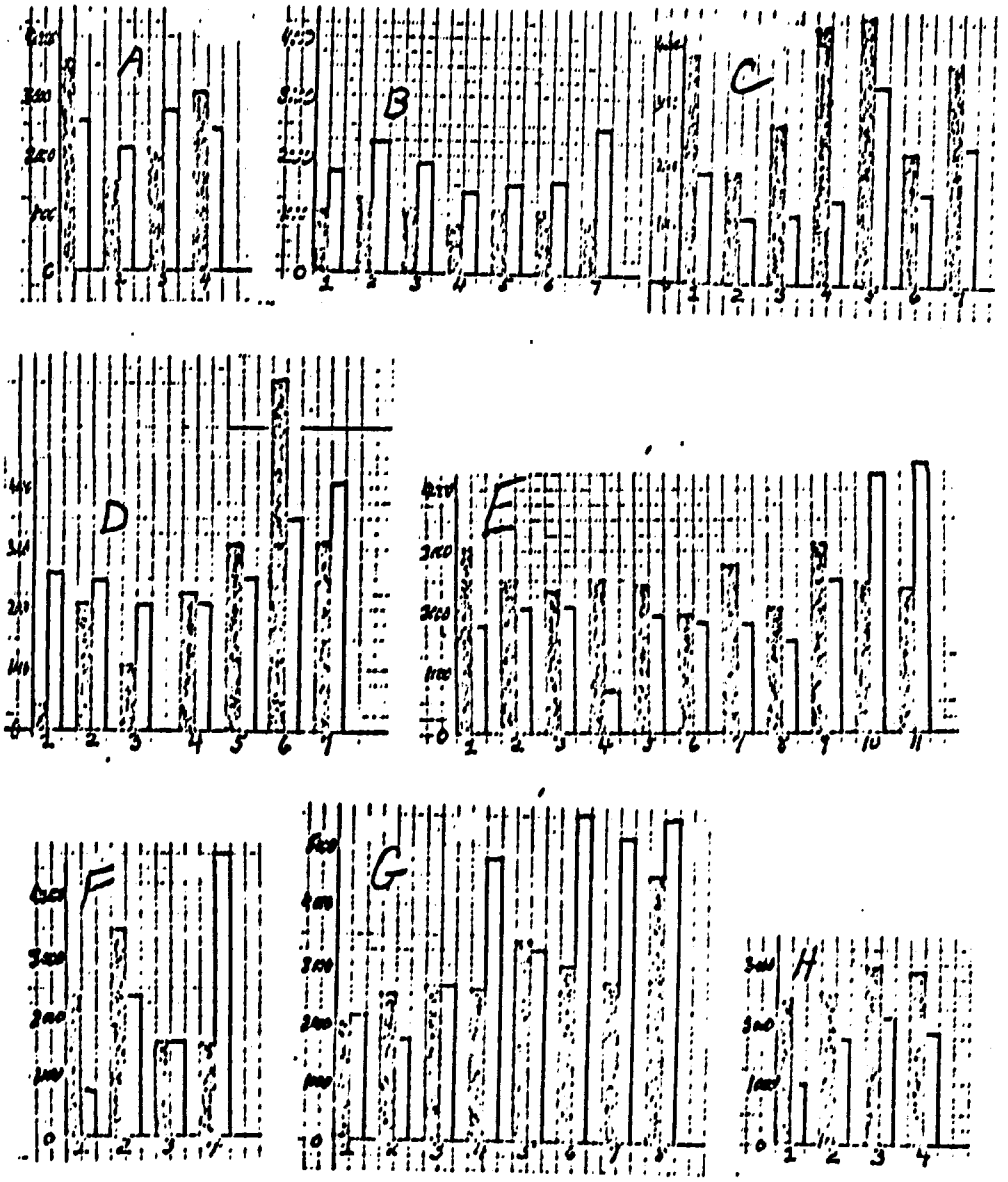

Figure 1. Results of 8 experiments showing average cercarial production per snail per collecting day from exposures to 5 (shaded) or 8 to 10 (white) miracidia. Ordinate = number of cercariae; abscissa = serial biweekly cercarial collections.

Death rates of the snails, both during prepatency and patency of the snail infections, were slightly lower in the 5- than in the 8-10-miracidium snails (Table I).

Table I. Percentage of Prepatent and Patent Deaths among Snails Exposed to 5 or 8-10 Miracidia Each.

REPLICATION	PREPATENT DEATHS		DEATHS/CERCARIAL COLLECTION DAY	
	5M	8-10M	5M	8-10M
1	—	—	8(0-16)	14(0-22)
2	4	12	5(0-25)	19(4-38)
3	2	4	12(0-23)	13(0-20)
4	8	39	11(4-43)	11(0-31)
5	6	14	11(0-25)	11(0-33)
6	20	13	2(0-6)	4(0-10)
7	20	10	9(0-32)	17(8-32)
8	12	14	6(0-12)	4(0-8)
Overall	10	15	8	12

The levels of snail exposure had no appreciable effect on the percentage of exposed snails which became infected and produced cercariae (Table II).

Table II. Percentage of Snails which Became Infected after Exposure to 5 or 8-10 Miracidia Each.

REPLICATION	% INFECTED SNAILS	
	5M	8-10M
1	—	—
2	75	91
3	86	78
4	78	68
5	57	44
6	48	76
7	78	90
8	66	81
Overall	70	75

DISCUSSION

Several facts emerged from this experiment: reducing the level of exposure of snails under these conditons did not appreciably change any of the parameters, although patent and prepatent death rates were a little lower in the 5-miracidium snails.

The finding that cercarial production was not affected by the number of miracidia used here for snail exposures is in line with the observation in this laboratory that as a rule not more than 2 primary sporocysts were seen in a snail whether exposed to 2 or to many miracidia. It appears that there is a mechanism for limiting the number of miracidia which develop concurrently into primary sporocysts in these snails.

CONCLUSIONS

(1) The means by which a limit is set on the number of primary sporocysts which develop concurrently in a snail should be explored. (2) The efficiency of our cercarial production would be improved by reducing the exposure level from 8-10 to 5 miracidia per snail, since a great deal of time and effort would be saved.

2. Epidemiological Differences in Intraspecific Geographical Strains of *Schistosoma mansoni*.

Suggestions have been accumulating that geographical strains of *S. mansoni* may differ in terms of epidemiology, morphology and virulence. We have tested epidemiological variations in two strains of this parasite with reference to cercarial production.

METHODS

The schistosome strain was maintained in the snail strain in which it was collected from the field, or in this strain mixed with another. Two strain associations were used: PR 1 schistosomes in M line *Biomphalaria glabrata*; and Nmri schistosomes in Nmri *B. glabrata*. Both of the schistosome strains and the M line snails were from Puerto Rico. PR 1 was collected in M line snails in the vicinity of Arecebo in 1950 and has been laboratory-maintained in the progeny of these snails since that time. The Nmri *S. mansoni* strain came from eggs in the stool of a Puerto Rican boy in a school in Washington, D. C. early in the 1940's. The Nmri *B. glabrata* snails are of mixed origin: to the susceptible pigmented snails brought originally from an unidentified location in Puerto Rico in the mid-1940's, was added a susceptible albino strain from NIH. The latter resulted from a cross of the susceptible pigmented PR 1 snail with a resistant albino snail from Brasil.

Snail exposure and maintenance conditions were the same for both strain associations. Snails were individually exposed to 8-10 miracidia each and kept at 27 ± 1C. After cercarial emergence began they were housed in the dark. They were tested individually for cercarial emergence from the 38th day postexposure by putting them in a brightly-lighted warm box at 33 to 34C from 7:30 to 10:30 A.M. Cercariae were collected once or twice a week under the same conditons. The total cercarial collection was calculated from counts of four 0.25 ml aliquots and the average number of cercariae per snail was recorded.

RESULTS

Data are graphed in Figure 2. Daily cercarial output by Nmri schistosomes in Nmri snails was about 3 times as high as that by PR1 schistosomes in M line snails throughout the 10 month comparison, except during May and the first 2 weeks of June. During this period, cercarial production by the PR1-M line combination increased almost to the level of that of the Nmri, but it was not sustained.

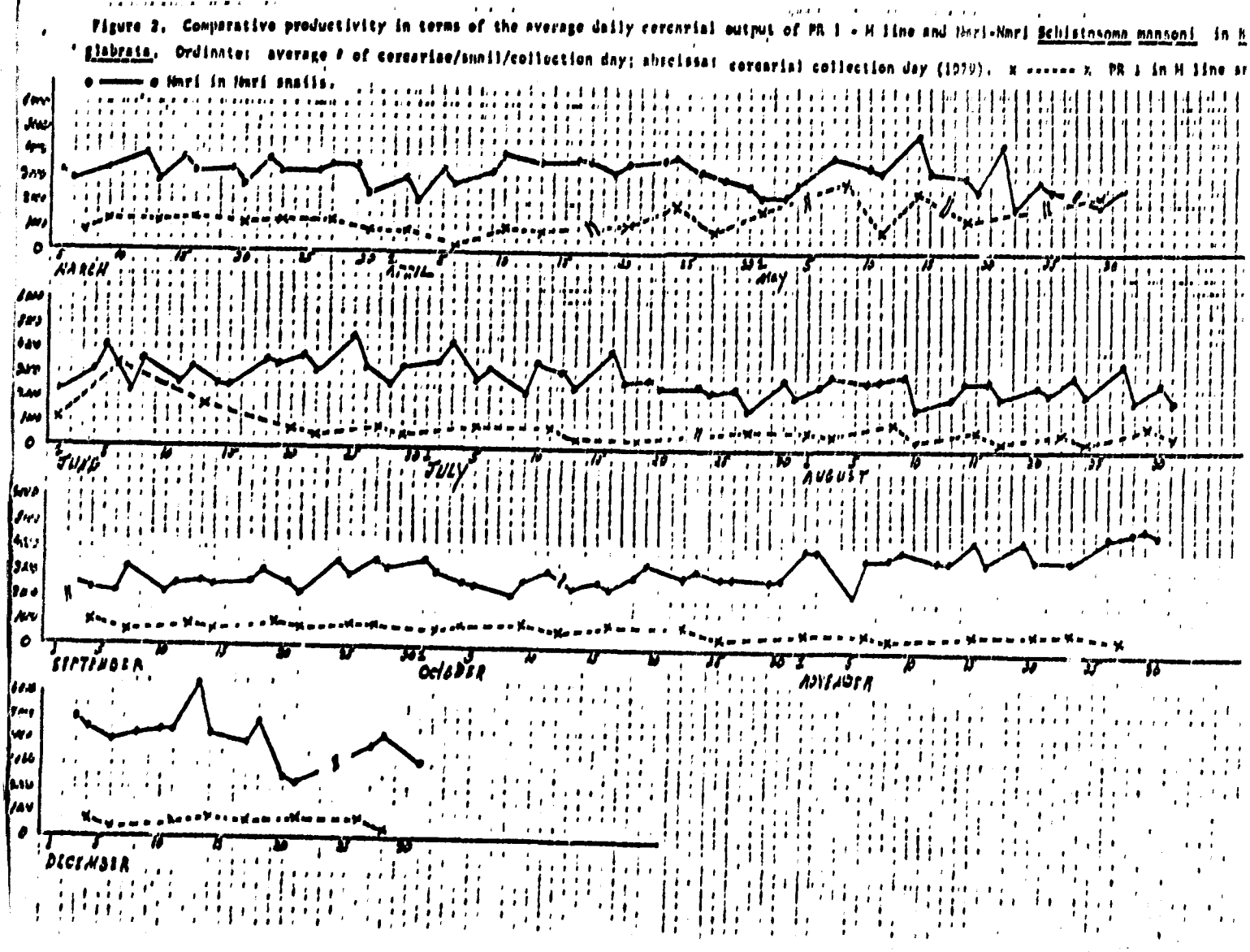

Figure 2. Comparative productivity in terms of the average daily cercarial output of PR 1 - M line and Nmri-Nmri Schistosoma mansoni in N
Biabrata. Ordinate: average # of cercariae/snail/collection day; abscissa: cercarial collection day (1979). x ------ x PR 1 in M line sr
o ——— o Nmri in Nmri snails.

DISCUSSION

No explanation is at hand either for the difference in productivity of cercariae by the two associations or for the temporary increase in cercarial output by the PR 1-M line association in the Spring. The fact that there was such an increase suggests that productivity might have been modified by environmental changes, but none has been recognized to have occurred. It seems more probable that the level of productivity is inherent in the association. Data should be recorded for other strain associations of *S. mansoni* and snails.

CONCLUSIONS

Cercarial productivity by various strain associations of schistosomes and snail hosts may vary. A baseline level of productivity should be established for the various parasite strains in their maintenance snail hosts.

8. Immunological Differences in Intraspecific Strains of *Schistosoma mansoni*

(with Drs. David Dean and Allen Cheever). Attempts are in progress in several laboratories, including our own, to develop an effective vaccine to protect man against infection with this parasite. It is essential to know whether intraspecific geographical strains will cross protect. Capacity for cross protection has been tested in several trials. If strains are cross protective, a monovalent vaccine will serve. If they are not cross protective, a polyvalent vaccine will be required. The capacity for cross protection has been tested preliminarily in several trials.

METHODS

In one series of experiments, 135 NMRI mice (NIH/NMRI (CV)) were vaccinated with about 500 cercariae which had been attenuated by irradiation with 50 kR of ^{60}cobalt. The cercariae were applied percutaneously to the mouse tails. Six to 8 weeks later the mice were challenged percutaneously with non-irradiated cercariae. Eight weeks later the worms were perfused from the mice and counted. All worms were from the challenge exposure, since the attenuated immunizing cercariae do not live to maturity. Schistosome strains used are shown in Tables 3 and 4. The numbers were compared with those from clean mice exposed concurrently with the challenge exposure of the vaccinated mice, and the percentage reduction was calculated. (Table 3).

In another series of experiments, protection afforded mice by a chronic infection was studied. A similar number of C57Bl/KsJ mice which had chronic infections of *S. mansoni* were challenged with 130 cercariae 10 weeks after the initial exposure. They were perfused 4 weeks after challenge. Worms of initial and challenge infections were identified by size. Worm burdens of experimental and control mice were compared as above. Strains used are shown in Table 4.

RESULTS

The tabulations (Tables 3 and 4) present the findings. As indicated by blanks in Table 3, experiments are still in progress. Diagonals show homologous strain protection.

Table III. Protection of NIH/NMRI (CV) Mice Vaccinated with Irradiated Cercariae of Five Different Intraspecific Strains of *Schistosoma mansoni* as Expressed by Percentage Reduction of Challenge Worm Burdens.

IMMUNIZING STRAINS	CHALLENGE STRAINS: PR I	PR II	T 15	EGYPTIAN	Nmri
PR I	46				43
PR II		66			63
T 15			61		51
Egyptian				49	54
Nmri					58

Table IV. Protection of C57Bl/KsJ Mice by Chronic Infections with Different Intraspecific Strains of *Schistosoma mansoni* as Expressed by Percentage Reduction of Challenge Worm Burdens.

IMMUNIZING STRAINS	CHALLENGE STRAINS: PRTA1C3	PRT13h	PRC13h
PRTA1C3	83	72	83
PRT13h	74	81	65
PRC13h	86	87	98

Greater protection was afforded the C57 black mice by chronic infections than the NMRI mice by vaccination, with the strains used. With chronic infections, homologous strain protection was a little greater than heterologous. With vaccination, heterologous strain protection was about the same as homologous, with the possible exceptions of the Egyptian and PR 1 strains.

This work is incomplete. Discussion and Conclusions seem unwarranted at this time. Indications are, however, that cross protection can be expected with some of the combinations.

4. Fine Structural Aspects of the Development of Artificially-derived Schistosomules of *Schistosoma mansoni*. (with Dr. Carolyn Cousin).

Our earlier work showed that transformation of penetrating cercariae (in vivo) into schistosomules was complete within 1 hour insofar as the features studied were concerned. In contrast, schistosomules produced by other means (in vitro) transformed more slowly (Cousin, Stirewalt and Dorsey, in press. *Experimental Parasitology*). It is our plan to set up a comparative time table describing transformation of the variously-derived schistosomules.

METHODS

Schistosomules were prepared by the appropriate artificial method and cultured in ELAC (lactalbumin hydrolysate in Earle's salts) at 37 C in CO_2/air for up to 5 days. At 1, 6, 24, 48, 96 and 120 hr., organisms were fixed, stained, sectioned and studied with EM as described in Annual Report No. 4 (FY 79). Their development was compared with that of in vivo postpenetration larvae.

Methods for schistosomule preparation were as follows. *In vivo* — cercariae which had penetrated mouse ear skin in situ were recovered from the excised skin after its maceration.

Rat skin — abdominal skin of young female rats was excised, the dermis removed, and the skin dried overnight in vacuo at room temperature. In a suitable system, cercariae were applied to the skin surface, allowed to penetrate and collected in ELAC as schistosomules (Stirewalt and Fregeau 1966).

Shear — cercariae were passaged 14 to 16 times through a No. 22 gauge injection needle fitted on a 10 ml syringe, and cultured in ELAC at 37 C for two hr. (Colley and Wikel 1974).

Centrifugation = cercariae were cooled, centrifuged, temperature-manipulated, Vortexed and cultured in ELAC at 37 C for 40 min. (Cazzinelli et al 1974).

Omnimix = cercariae were stirred in an Omnimix mixer modified by substitution of a blunted plastic blade for the metal one. Mixing was for 7 sec. at the medium setting. Organisms were incubated in ELAC at 37 C for 2 hr. (Dorsey and Cousin in press, Journal of Parasitology).

Rat serum = cercariae were centrifuged and incubated for 3 hr. in 50% rat serum in ELAC at 37 C (Eveland and Morse 1975).

Each of these experiments was done with at least 3 different pools of cercariae, using 20 to 50 cercariae each time. Organisms of each derivation were tested for stage of transformation by the parameters listed in Table 5. The fine structural descriptions which have been completed are outlined in Table 6.

RESULTS

Functional testing of the state of transformation of schistosomules derived artificially and comparison of them with in vivo postpenetration schistosomules and cercariae are tabulated (Table V). Data in the far right column and the bottom line have been added to the table since Annual Report No. 4. It will be noted that cercariae which penetrated skin in situ on a living host or in vitro as excised dried rat skin developed at essentially the same rate. Assessment of schistosomular status by the parameters listed in Table V confirms earlier indications that cercariae stimulated artificially to transform without skin penetration did so, but the changes occurred more slowly. Schistosomules of all derivations were able to mature after injection into mice.

Table V. Comparative Characteristics of Schistosomules of the Various Derivations as Tested by the Parameters Listed.

Parameter	Cerc		In vivo	Rat Skin	Shear	Centrif.	Omnimix	Rat Serum
Water Intolerance	No		<15 min	<1 hr	72 hr	<3 hr	>72 hr	<1 hr
Gland Exhaustion	No		<1 hr	3 hr	72 hr	48 hr	72 hr	3 hr
Cilia Capability	100%	1 hr	1%	7%	86%	54%	90%	20%
		24 hr	"	1%	32%	20%	26%	5%
Infectivity after Injection	29%	1 hr	6%	40%	7%	5%	13%	8%
		24 hr	19%	30%	13%	9%	20%	4%

Tabulation of the schedule of development in in vivo schistosomules (in skin after penetration) is complete in Table VI, except for two additional aspects: tegumental heptalamination and change of cell nuclei from cercarial heterochromasy to the euchromasy characteristic of schistosomules. Both occurred in in vivo schistosomules within 1 hr.

Study of the fine structural changes in artificially-derived schistosomules which were cultured in vitro through 5 days, was delayed by the finding that our culture medium was inadequate to support the organisms. Transforming cultured organisms began to show vesiculation within 6 hr. and most died within the 5 day culture period. A new and satisfactory culture system, that of Dr. Paul Lasch, has been adopted. Sections of shear pressure and centrifuged, room temperature schistosomules cultured through 5 days are in process of study. Shear pressure schistosomules followed the transformational schedule of in vivo schistosomules more closely than those of other artificial derivations except rat skin organisms. They paced in vivo transformation as assessed by heptalamination of the surface membrane (1 hr.), tegumental infolding (48 hr.), and tegumentally directed extrusion of cyton granules (1 hr.). Change from heterochromatic to euchromatic nuclei, however, was delayed in the artificially derived organisms. This did not occur until 48 hr. in shear pressure schistosomules as compared with 1 hr. in in vivo.

From the wealth of morphological description of transformation of cercariae to schistosomules in vivo (Table VI), several parameters have been selected as critical indicators of transformation. These will be emphasized in the continued comparison of later development of artificially-derived schistosomules: surface membrane heptalamination; tegumental infolding; modification of the glycocalyx as indicated by loss of CIIR capacity (the Ag/Ab reaction which results in the seroenvelope around cercariae); and the nuclear change from hetero- to euchromasy.

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Table VI. Tabulation of the Sequence of Changes in Cercariae Changing to Schistosomules after Penetration of Mouse Ear Skin.

ORGANISMS	ANTERIOR ORGAN (ORAL AREA): [illegible]	ANTERIOR ORGAN: HEAD GLAND	ANTERIOR ORGAN: HEAD GLAND	ANTERIOR ORGAN: HEAD GLAND	ANTERIOR ORGAN: PENETRATION GLANDS (DUCTS) PRE	ANTERIOR ORGAN: PENETRATION GLANDS (DUCTS) POST	BODY PROPER (MIDDLE AND ABORAL): DIGESTIVE SYSTEM [illegible]	BODY PROPER: DIGESTIVE SYSTEM [illegible]	BODY PROPER: PENETRATION GLANDS PRE	BODY PROPER: PENETRATION GLANDS POST	BODY PROPER: PENETRATION GLANDS PRE	BODY PROPER: PENETRATION GLANDS POST	EXCRETORY SYSTEM: COLLECTING DUCTS MAIN	EXCRETORY SYSTEM: COLLECTING DUCTS SMALL	NERVOUS SYSTEM: NEUROPILE	NERVOUS SYSTEM: [illegible]	[illegible]	[illegible]	[illegible]	[illegible]
1 HOUR	Granule filled	Collapsed	Granule filled	Variable	Granule filled	Empty of granules	Constricted lumen & granules in epith. lumen	Alternating constriction & dilation	Variable	Variable	Variable	Variable	Dilated	/ Constricted	Intact	Some were damaged	Influx of cells in aboral end	Granule filled	Granule filled	Variable, some empty of granules
6 HOURS	Granule filled	Collapsed	Granule filled	Variable	Granule filled	Empty of granules	Constricted lumen & granules in epith. lumen	Alternating constriction & dilation	Variable	Variable	Variable	Usually empty of granules but variable	Dilated	A few dilated	Intact	Some were damaged	Influx of cells in aboral end	Granule filled	Few granules	Variable, some empty of granules
24 HOURS	Granule filled	Collapsed	Granule filled	Empty of granules	Granule filled	Empty of granules	Alternating constriction & dilation	Alternating constriction & dilation	Empty of granules	Empty of granules	Empty of granules	Empty of granules	Dilated	Many dilated	Intact	Intact	Influx of cells in aboral end	Granule filled	Few granules	Variable, some empty of granules
48 HOURS	Variable	Collapsed	Variable	Empty of granules	Variable	Empty of granules	Alternating constriction & dilation	Alternating constriction & dilation	Empty of granules	Empty of granules	Empty of granules	Empty of granules	Dilated	Many dilated	Intact	Intact	Influx of cells in aboral end	Few granules, elevated ridges with numerous holes	Few granules	None
72 HOURS	Variable	Collapsed	Variable	Empty of granules	Usually empty	Empty of granules	Alternating constriction & dilation	Alternating constriction & dilation	Empty of granules	Empty of granules	Empty of granules	Empty of granules	Dilated	Many dilated	Intact	Intact	Influx of cells in aboral end	Few granules, elevated ridges with numerous holes and vacuoles on surface	Few granules	None
96 HOURS	Variable	Collapsed	Variable	Empty of granules	Empty of granules	Empty of granules	Alternating constriction & dilation	Alternating constriction & dilation	Empty of granules	Empty of granules	Empty of granules	Empty of granules	Dilated	Many dilated	Intact	Intact	Influx of cells in aboral end	Few granules, elevated ridges with numerous holes and vacuoles on the surface	Few granules	None
120 HOURS	Variable	Collapsed	Variable	Empty of granules	Empty of granules	Empty of granules	Alternating constriction & dilation	Alternating constriction & dilation	Empty of granules	Empty of granules	Empty of granules	Empty of granules	Dilated	Many dilated	Intact	Intact	Influx of cells in aboral end	Few to many granules, elevated ridges with numerous holes and vacuoles on surface	Few granules	None
CERCARIA	Cercarial type granule only	Intact	Few granules	Few granules	Granule filled	Granule filled	Constricted	Dilated	Granule filled	Granule filled	Granule filled	Granule filled	Dilated	Many dilated	Intact	Intact	Few observed in aboral area, instead the pre & post acetabular glands fundus were seen.	Cercarial type granules only	Many granules	No granules present

In the course of the year, two new procedures have been developed which have contributed materially to this work. One is a simplified way to induce cercariae to transform to schistosomules. Cercariae were centrifuged x 1000g at room temperature for 7 min., the supernate discarded and the organisms incubated in ELAC at 37 C for 3 hr. They remained tailed, but the bodies satisfied the requirements for schistosomules.

The other new procedure is an additional way to distinguish schistosomules from cercariae, i.e., by freezing. It has not been possible to freeze cercariae, store them in liquid nitrogen (-196 C), thaw them and recover any in a viable state. By contrast, about 90% or more of schistosomules so processed are viable; up to 70% are essentially normal in appearance and movement.

DISCUSSION

Apparently, cercariae transformed to schistosomules under a variety of conditions, for all the methods tested produced transformed organisms. Transformation progressed, however, at different rates with the various methods. In terms of the 3 parameters used in Table VII, the cercariae changed fastest after penetration of skin, whether in situ on a living host or in vitro through dried rat epidermis.

Table VII. Ranking of Variously-derived *Schistosoma mansoni* Schistosomules According to the Speed with which Three Specific Changes Occurred.

WATER INTOLERANCE	LOSS OF CHR CAPABILITY	GLAND EXHAUSTION
In vivo	In vivo	In vivo
Rat skin & rat serum	Rat skin	Rat skin & rat serum
Centrifuge/ vortex	Rat Serum	Centrifuge/ vortex
Shear	Centrifuge/ vortex	Shear & Omnimix
Omnimix	Shear & Omnimix	

That schistosomules were eventually produced by all methods studied, is important. When this assessment is complete, methods of cercarial conversion can be chosen rationally in terms of the type of organism needed. Two more types of schistosomules remain to be examined: those produced (1) over skin surface lipid and (2) after centrifugation only.

The obvious next phase is a sorting out of the steps in each method, to identify the transformation trigger and to describe the responses of the organisms and the biological mechanisms of change. Clarification of these may open new avenues of control of the disease by preventing transformation of cercariae to schistosomules.

CONCLUSIONS

Schistosomules may be produced from cercariae by many different methods. Rates of transformation vary with the method. The key to the transformation trigger (s) and the organisms' mechanisms of response should be identified.

SIGNIFICANT ACCOMPLISHMENTS

1. The most efficient exposure level for snails, using Nmri strains of *Schistosoma mansoni* and *Biomphalaria glabrata*, was established for our conditions. It was 5 miracidia pe. snail. Cercarial output was not increased by raising the exposure level.

2. Intraspecific strains of *S. mansoni* were found to vary in cercarial productivity. PR 1 *S. mansoni* in M line *B. glabrata* provided only about 1/3 as many cercariae/snail/day as Nmri parasites in Nmri snails under the same conditions.

3. Cross protection by intraspecific strains of *S. mansoni* was demonstrated in mice. Among the strains used for immunization and challenge, there was some variability in the level of protection. Greater protection was afforded mice by a chronic infection than by vaccination with irradiated cercariae.

4. Cercaria-schistosomule transformational morphological changes have been described in detail under natural conditons. These changes occurred in the parasite surface, tegument, tegumental secretory cells, body cell nuclei and digestive tract.

5. A new simpler method of producing schistosomules has been developed. It consists merely of centrifuging cercariae and incubating them for 3 hr. at 37 C in a culture medium.

6. An additional criterion for distinguishing schistosomules from cercariae has been shown to be the capacity of schistosomules to recover after storage in liquid nitrogen (-196 C). Cercariae handled similarly die.

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PLANS FOR THE FUTURE

1. Continue EM study of the morphological changes occurring in vitro from 24 to 120 hr. as cercariae transform to schistosomules after artificial stimulation.
2. Describe chemically and biologically the anticercarial effect of rotifer-conditioned water.
3. Continue study of the most efficient techniques for high-level production of *Schistosoma mansoni* cercariae.
4. Test an in vitro model system for studying mechanisms of cercarial penetration: Nitex screens or other suitable artificial substrates.
5. Analyze the transformation trigger and the parasite reaction involved in the cercaria-schistosomule conversion.
6. Expand the cross protection studies using cercariae of other strains than PR1 for the immunizing strain.

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